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14. ABSTRACT

The impact of osteoporosis on individual patients and their families, health care systems, and national economies is enormous. At the onset of menopause, lack of estrogen and other sex steroids results in increased bone turnover and net bone loss. Fortunately, only 25-30% of post-menopausal women will develop vertebral fractures – the hallmark of post-menopausal osteoporosis – because of differences in the amount of bone lost.

This proposal presents a novel theory to explain why only some women get osteoporotic fractures and proposes a first experiment to test this theory. The theory suggests a critical link between the estrogen loss, the advanced glycation endproduct (AGE) content of the bone, and signaling via the receptor for advanced glycation endproducts (RAGE) and that the combination of these three components determines the extent of bone loss post menopause.

The broadly accepted ovariectomized female rat was used to model post-menopausal osteoporosis. Some of these rats were fed a special diet to induce advanced glycation endproduct formation. Their blood serum was analyzed to confirm AGE formation, bone turnover and RAGE expression. Bone morphometry was measured with microCB and histomorphometric techniques. Oseteclast expression of RAGE and co-localization with AGEs in the bone matrix was tested with immunohistochemistry.

15. SUBJECT TERMS
postmenopausal osteoporosis, ovariectomy, estrogen loss, advanced glycation endproducts, receptor for advanced glycation endproducts, bone remodeling, bone mineral density, high fructose diet

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Why do only some women develop post-menopausal osteoporosis?

1. Introduction

At the onset of menopause, lack of estrogen and other sex steroids results in increased bone turnover and net bone loss. However, only 25-30% of post-menopausal women will develop vertebral fractures – the hallmark of post-menopausal osteoporosis. This proposal presents a novel theory to explain why some women get osteoporotic fractures and proposes a first experiment in the ovariectomized (OVX) female rat model. A decrease in estrogen levels (down from pre-menopausal levels) increases the multi-ligand Receptor for Advanced Glycation Endproducts(RAGE) expression in osteoclasts. This heightened RAGE expression stimulates osteoclasts to resorb bone and to express more RAGE in proportion to the Advanced Glycation Endproducts (AGE) content of the bone creating a positive feedback loop. There is natural variation between women post-menopause in the amount of estrogen lost and in the AGE content of their bones. Therefore, these two factors may determine whether women will develop osteoporotic fractures due to bone mass loss determined by the critical receptor for advanced glycation endproducts (RAGE). To test the effect of estrogen loss on RAGE expression of osteoclasts, the established female rat OVX model of post-menopausal osteoporosis was selected. To test the effect of pre-existing AGE content in bones of the rat model, a high fructose diet was chosen. High fructose diet has been shown to produce increased levels of AGEs (pentosidine, carbodymethyllysine, general AGE-linked fluorescence) in tissues of the rat including bone. The current study aims to investigate if a high amount of bone loss is correlated with elevated AGE content, and whether the loss of estrogen resulting from menopause leads to an increased expression of RAGE. We hypothesize that:

1) The amount of bone lost due to OVX is higher when bone AGE content is higher.
2) OVX of female rats will increase osteoclast RAGE expression.

2. Keywords

Post-menopausal osteoporosis, ovariectomy, estrogen loss, advanced glycation endproducts, receptor for advanced glycation endproducts, bone remodeling, bone mineral density, high fructose diet

3. **Accomplishments**

Statement of Work (SOW) had 6 tasks which were all accomplished

Task 1: Apply for and receive Animal Use Protocol approval
We received the approval both from the University of Toronto ethics committee and from Acuro

Task 2: Order and delivery of 60 three-month old female rats
The rats were randomly assign rats to the six experimental groups (n = 10 each group)

Task 3: Experimental Period 1 – all six groups of 10
- 3a. Take time-zero baseline blood samples from all sixty rats
- 3b. Start the controlled research diets - Normal diet groups (3x n of 10) and High Fructose Diet groups (3x n of 10
- 3c. at the end of Period 1, euthanize one Normal diet group (n=10) and one High Fructose Diet group (n=10) to characterize the effect of the High Fructose Diet.

3c.1. Faxitron scans, collect blood and bone specimens

Task 4: Experimental Period 2 – remaining four groups of 10
- 4a. Perform ovariectomy or sham operations on the four remaining groups
- 4b. All four groups continue on Normal Diet (months 7-9)
- 4c. 4 weeks after ovariectomy and sham operations, collect blood samples
- 4d. Euthanize four remaining groups (Normal diet + Sham, Normal Diet + OVX, HFD + Sham, HFD + OVX)

4d.1. Faxitron scans, collect blood and bone specimens

Task 5: Data Acquisition and Analysis
- 5a. MicroCT scanning and analysis
- 5b. Preparation of histomorphometry specimens
- 5c. Histomorphometry data collection and analysis
- 5d. Blood and Bone Biochemistry
- 5e. Histology and Immunohistochemistry

Task 6: Finalization and Dissemination
- 6a. Data analysis
- 6b. Report and thesis writing

Hypothesis and Specific Objectives

1) The amount of bone lost due to OVX is higher when bone AGE content is higher.
2) Bilateral ovariectomy (OVX) of female rats will increase osteoclast RAGE expression.

Objective 1:
Using a high fructose diet model, determine if AGE bone and serum content increases and if the amount of bone loss in the OVX rat model is higher when the pre-existing AGE content of the bones is higher.

Objective 2:
Using the established female rat OVX model, determine if OVX and the resulting loss of estrogen lead to an increased osteoclast expression of RAGE.

Major Findings

***In vivo* rat model:**

Sixty three-month old female Wistar rats were acquired from Charles River Laboratories. After allowing for a two-day adjustment period the rats were spilt into six groups of ten. The first three groups were fed a high fructose diet that contained 60% fructose (HF) from Harlan Laboratories (TD 89247) and the remaining three groups were fed an isocaloric control diet (CD) that contained only 5% fructose (TD 86489). The rats continued on their respective diets for three months then one group from each diet was sacrificed to establish baseline parameters, one group from each diet underwent ovariectomy procedures (OVX) in which both of the ovaries were surgically removed to simulate post-menopausal bone loss, and one group from each diet underwent a sham-operation (SHAM) in which their ovaries were exposed but not excised. The four remaining surgical groups continued on a maintenance diet from Harlan Laboratories for three more months, in which time the OVX groups were allowed to lose bone and at nine months of age all of the surgical groups were sacrificed. Ten and three days before each sacrifice calcein green was injected intrapertionally for analysis of histomorphometric measurement of bone formation (Table 1).

Group number	Group 1: HFD	Group 2: HFD	Group 3: HFD	Group 4: Control Diet	Group 5: Control Diet	Group 6: Control Diet
Month 1	1 ml blood sample from tail vein	1 ml blood sample from tail vein	1 ml blood sample from tail vein	1 ml blood sample from tail vein	1 ml blood sample from tail vein	1 ml blood sample from tail vein
Month 3	OVX 1 ml blood sample from tail vein	OVX-SHAM 1 ml blood sample from tail vein	Euthanasia 1 ml cardiac blood sample	OVX 1 ml blood sample from tail vein	OVX-SHAM 1 ml blood sample from tail vein	Euthanasia 1 ml cardiac blood sample
Month 3	Begin maintenance diet	Begin maintenance diet	X	Begin maintenance diet	Begin maintenance diet	X
Month 4	1 ml blood sample from tail vein	1 ml blood sample from tail vein	X	1 ml blood sample from tail vein	1 ml blood sample from tail vein	X
Month 6	Euthanasia 1 ml cardiac blood sample	Euthanasia 1 ml cardiac blood sample	X	Euthanasia 1 ml cardiac blood sample	Euthanasia 1 ml cardiac blood sample	X

Table 1. Animal study plan.

Blood was removed from the tail veins of each rat at four time points throughout the study; the first was before the rats were started on their respective diets, the second was during the first sacrifice and surgeries. The third blood extraction was performed one month after surgeries, and the final was at final sacrifice. Serum was isolated from the blood through centrifuging, and was stored for later analysis.

Bone Quality Assessment

Method:
Micro-computed tomography (microCT) and dual-energy x-ray absorptiometry (DXA) were used to investigate specimen morphometry and density. To look at cortical bone, the left femur was scanned at the mid-diaphysis in microCT and the mean cross-sectional area (mm^2), mean major and minor diameter (mm), cortical thickness (mm), cortical porosity(%) and volumetric bone mineral density (vBMD) were recorded. The bone mineral density (BMD) and bone mineral content (BMC) was also measured using DXA scanning of the same femur. To look at trabecular bone, the sixth lumbar vertebrae was scanned using microCT and the vBMD, trabecular bone volume (BV/TV; %), trabecular thickness (Tb.Th; μm), trabecular separation (Tb.Sp.; μm), and trabecular number (Tb.N.; 1/mm) were recorded. We also measured the bone mineral density and bone mineral content using DXA of the same vertebrae.

Results:
Femurs and vertebrae measured from rats sacrificed at 6 months of age (before any surgical procedures) showed no changes in cortical or trabecular bone mineral density or microarchitecture. Femurs measured from the rats that underwent the OVX and SHAM surgeries showed no difference in cortical microarchitecture (Table 3), while the vBMD of the OVX groups were greater then the SHAM groups (Fig 1) This result is common, as OVX groups sometimes have periosteal bone growth which can lead to a higher vBMD (Turner *et al*., 1987 & Miller *et al*., 1991). In addition, no difference in BMD or BMC was found in cortical bone through DXA scanning. The vertebrae scanned by microCT showed a decrease in trabecular bone volume, number and density due to OVX alone; which is to be expected of this model (Kalu, 1991). DXA analysis also showed a decrease in BMD due to OVX alone (Fig 3), however BMC was unchanged.

	HF+OVX	HF+SHAM	CD+OVX	CD+SHAM
vBMD	1.0097± 0.0221*	0.9897 ± 0.04085	1.0133± 0.06882*	0.9440 ± 0.06045
Cross Sectional Bone Area	7.5237± 0.59034	7.1634± 0.47340	7.3508± 0.50325	7.2869± 0.47607
Cortical Thickness	0.8029± 0.04619	0.8040± 0.03627	0.7878± 0.03586	0.8315± 0.03448
Mean Major Diameter	4.489± 0.1980	4.380 ± 0.2326	4.548 ± 0.2450	4.476± 0.2148
Mean Minor Diameter	3.231± 0.1482	3.233± 0.1388	3.229± 0.1252	3.178± 0.1724

Table 2. MicroCT results of femur scanned at mid-diaphysis
*Values reported as mean ± standard error. * Significant ($p<0.05$) compared to SHAM controls*

Fig 1. MicroCT results of femur at mid-diaphysis scanned at 11.6µm voxel size. Mean ± SD, n=9, two-way ANOVA, Surgery p=0.031, diet p=0.297, interaction p=0.222.

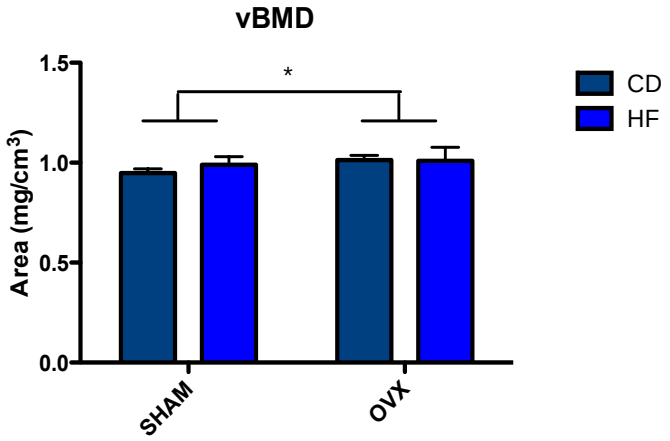


Fig 2. MicroCT results of secondary spongiosa in 6th lumbar vertebrae scanned at 11.6µm voxel size. Mean ± SD, two-way ANOVA, n=9. (A) surgery p=0.019, diet p=0.311, interaction p=0.271; (B) surgery p=0.000, diet p=0.128, interaction p=0.418; (C) surgery p=0.000, diet p=0.234, interaction p=0.766.

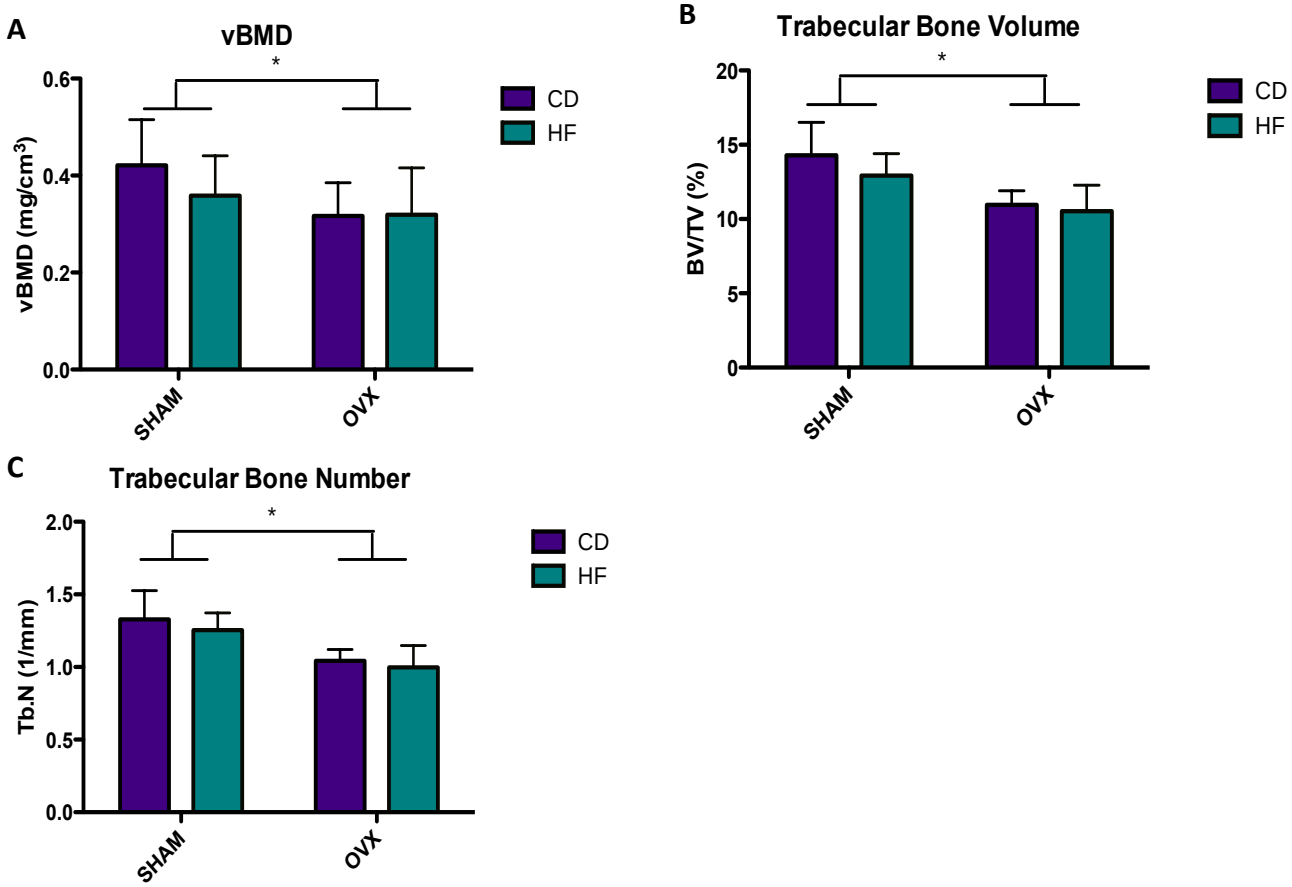
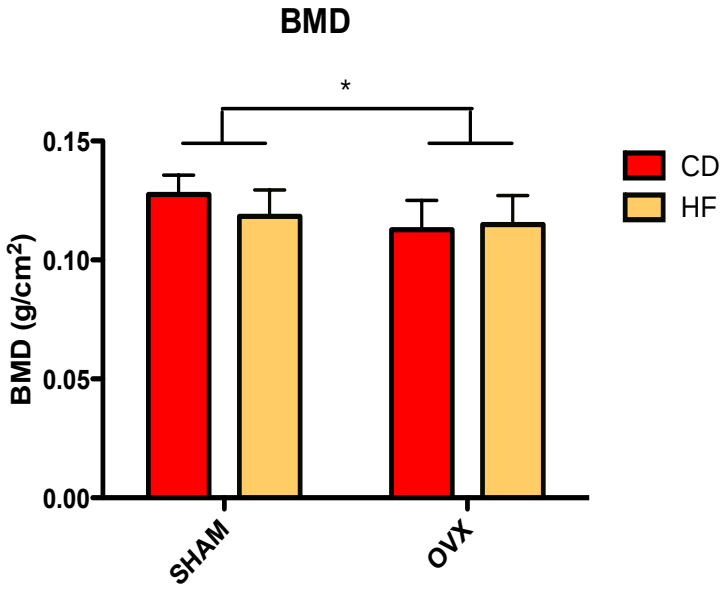


Fig 3. DEXA results of secondary spongiosa in 6th lumbar vertebrae. Mean ± SD, two-way ANOVA, n=9, surgery p=0.0155, diet p=0.3382, interaction p=0.1191.



Liver Histology

Method:
Sections of the livers were removed and fixed in 10% neutral buffered formalin for 48 hours then processed and embedded in paraffin wax. 5-micron sections were cut and stained by hematoxylin and eosin to determine whether the high fructose diet caused the rats to develop non-alcoholic fatty liver disease.

Results:
The liver histology showed no signs of development of non-alcoholic fatty liver disease in any groups fed the control diet or the high fructose diet.

Bone Histology (decalcified)

Method:
The right proximal tibia was fixed in 10% neutral formalin buffer for 5 days, then decalcified for 4 weeks in 0.5M EDTA in Tris buffer. They were then processed and embedded in paraffin wax. 5-micron sections were cut and stained for tartrate resistant acid phosphatase (TRAP). TRAP is an enzyme expressed in active osteoclasts and its expression is strongly correlated to the resorptive activity of osteoclasts (Angel *et al.,* 2000). For this assay we measured osteoclast surface (Oc.S) and the number of active osteoclasts (N.Oc) and we normalized both values to bone surface (Oc.S/BS, N.Oc/BS).

Results:
No significant differences were noted between the groups that were sacrificed at 6 months of age nor amongst the surgical groups in either parameter. However, the interaction of high fructose diet and OVX surgery (HF+OVX) showed a trend towards having the greatest Oc.S/BS (p=0.069) and N.Oc/BS (p=0.07) (Table 4). OVX rats experience a biphasic bone loss; first is the rapid phase of bone loss which increases osteoclastic surface for the first 100 days after surgeries, then experience a steady state of bone loss, where bone turnover is subsided but still at osteopenic levels (Wronski *et al*., 1989). In our studies we allowed our rats to lose bone for 3 months, so we may have missed the period where osteoclasts were most active, and therefore expressing the most amount of TRAP. This is clearly evident in the CD+OVX group, whose levels of Oc.S/BS and N.Oc/BS are similar, if not less then, the SHAM-operated groups. However, in the HF+OVX, this decrease is not experienced and the levels of both these parameter stay elevated. This possibly suggests that the high fructose diet in combination with the OVX surgery is either having some sort of preventative effect against a decrease in the activity of the osteoclasts, or is causing the maturation of more osteoclasts, even in the slow phase of bone loss.

	HF+OVX	**HF+SHAM**	**CD+OVX**	**CD+SHAM**
Number of osteoclasts (#)	64.22 ± 49.11	80.89 ± 38.1	43.75 ± 21.63	120.1 ± 39.27
Osteoclast Surface (mm)	7.52 ± 0.59	7.16 ± 0.47	7.35 ± 0.5	7.29 ± 0.48
Number of osteoclasts/bone surface (1/mm)	1.05 ± 0.42*	0.77 ± 0.35	0.76 ± 0.22	0.87 ± 0.19
Osteoclast surface/bone surface (%)	0.07 ± 0.05*	0.06 ± 0.04	0.05 ± 0.14	0.07 ± 0.02

Table 3. Results of histomorphometry analysis of TRAP stained sections for surgical groups
*Values reported as mean ± SD. *Trend (0.07>p>0.05) interaction of HF+OVX having greatest amount.*

Bone Histology (undecalcified)

Method:
The left proximal tibia was fixed in 70% ethanol for 5 days, then dehydrated in ascending concentrations of acetone and infiltrated with Spurr resin under vacuum. They were then embedded in Spurr resin and 7-micron sections were cut for static and dynamic histomorphometry.

For static histomorphometry, one section was stained with Goldner's Trichrome stain to allow for the measurement of the osteoid seam. For this assay we measured the osteoid volume (OV), osteoid surface (OS) and osteoid width (O.Wi), the former two we normalize to bone volume and bone surface, respectively (OV/BV, OS/BS).

Results:
No significant differences were found in rats sacrificed at 6 months of age. However, for the surgical groups, the OV/BV and OS/BS were significantly increased in groups that underwent OVX, while diet did not have an effect (Table 6). These results are consistent with previous reports of histomorphometry in OVX rats (Wronski *et al.*, 1989). An interesting result was that the high fructose diet alone caused a significant increase in O.Wi. Past studies have suggested that osteoid width may be correlated with osteoblast activity, so this may indicate that there was increased osteoblast activity due to the diet (Parfitt *et al.*, 1995). However, this was the only bone formation parameter that was significantly affected by the high fructose diet, so it unlikely that the diet caused a dramatic increase in osteoblast activity.

Method:
For dynamic histomorphometry, a sequential section was left unstained and was viewed under UV light to detect calcein green labeling. Calcein green is a fluorochrome that strongly chelates to calcium ions and thus is incorporated into bone during mineralization. It is often used as a surrogate measurement for bone formation. The calcein lines were quantified to obtain the following parameters: single label surface (sLS, mm); double label surface (dLS, mm); mineralizing surface (MS, mm); and interlabel width (Ir.L.Width, μm). Normalized parameters obtained were: percent mineralizing surface (MS/BS, %); mineral apposition rate (MAR, um/day, formula=interlabel width/labeling period); bone formation rate normalized over bone surface (BFR/BS, μm/day/mm, formula=MS*MAR/BS); and bone formation rate normalized over bone volume (BFR/BV, $\mu m/day/mm^{2,}$, formula= MS*MAR/BV).

Results:
There was no significant difference in dynamic bone formation between the groups sacrificed at 6 months of age. However, for the surgical groups, results showed that OVX surgeries alone led to significant increases in dynamic parameters of bone formation including dLS, Ir.L.Wodth, MS/BS, MAR, BFR/BS, BFR/BV (Table 6). These results are consistent with previous reports of dynamic histomorphometry performed on OVX rats (Wronski *et al.*, 1989).

	HF+OVX	HF+SHAM	CD+OVX	CD+SHAM
Osteoid Surface (mm)	1.68 ± 2.28*	0.78 ± 0.65	1.57 ± 1.02*	0.38 ± 0.34
Osteoid Volume (mm^2)	0.02 ± 0.03	0.007 ± 0.005	0.009 ± 0.01	0.003 ± 0.005
Osteoid Surface/Bone Surface (%)	0.05 ± 0.05*	0.01 ± 0.01	0.04 ± 0.03*	0.005 ± 0.004
Osteoid Volume/ Bone Volume (%)	0.008 ± 0.008*	0.002 ± 0.001	0.006 ± 0.003*	0.0008 ± 0.0008
Osteoid Width (µm)	7.55 ± 3.01**	10.15 ± 4.57**	5.94 ± 1.79	5.29 ± 5.29

Table 4. Results of histomorophometry on trichrome stained sections from surgical groups. *Values reported as mean ± SD. * Significant ($p<0.05$) compared to SHAM controls. **Significant ($p<0.05$) compared to CD.*

	HF+OVX	HF+SHAM	CD+OVX	CD+SHAM
Single Label Surface (mm)	6.84 ± 3.6*	13.24 ± 4.23	7.43 ± 3.16*	13.62 ± 5.92
Double Label Surface (mm)	5.20 ± 3.48*	3.8 ± 1.77	5.21 ± 2.29*	1.83 ± 1.14
Mineralizing Surface (mm)	8.62 ± 3.78	10.42 ± 3.29	8.92 ± 2.95	8.64 ± 3.88
Interlabel Width (µm)	25.74 ± 4.27*	23.01 ± 1.73	24.97 ± 3.38*	21.82 ± 2.92
Mineralizing Surface/Bone Surface (%)	0.29 ± 0.17*	0.18 ± 0.14	0.26 ± 0.14*	0.12 ± 0.06
Mineral Apposition Rate (µm/day)	3.68 ± 0.61*	3.29 ± 0.25	3.57 ± 0.48*	3.12 ± 0.42
Bone Formation Rate/ Bone Surface (µm/day/mm)	1.07 ± 0.65*	0.61 ± 0.5	0.94 ± 0.56*	0.39 ± 0.21
Bone Formation Rate/ Bone Volume ($\mu m/day/mm^2$)	22.41 ± 15.25*	9.36 ± 6.5	19.83 ± 12.31*	9.65 ± 6.82

Table 5. Results of dynamic histomorphometry in surgical groups. *Values reported as mean ± SD. * Significant ($p<0.05$) compared to SHAM controls.*

Blood Biochemistry

Method:
The blood serum from each time point was analyzed using anti-rat ELISA kits to investigate the amount of circulating AGEs (pentosidine and carboxymethyllysine (CML)) and soluble RAGE.

Results:
Circulating pentosidine was present, yet did not differ between each of the surgical groups at any time point. Circulating CML, was present at each time point, but only showed a difference one month after surgeries in which the two surgical groups that had been fed the control diet showed an increase level of serum CML (Figure 4). From this we can conclude that the high fructose diet alone did not have a detectable effect on the levels of circulating AGEs, however the control diet caused an increase in CML levels, which was a surprising and unexpected result.

Soluble RAGE was also present in each surgical group at each time point, and only showed a difference one month after surgeries, in which OVX groups had higher amounts circulating RAGE (Figure 5). Soluble RAGE is thought to act as a decoy receptor to blunt intracellular signaling triggered by ligation of RAGE (Xie *et al.*, 2013). Therefore it is possible that soluble RAGE was increased following OVX to help regulate RAGE effects.

Fig 4. ELISA results for serum CML measured one month after surgeries. Mean ± SD, n=9, two-way ANOVA, surgery p=0.162, diet p=0.019, interaction p=0.216.

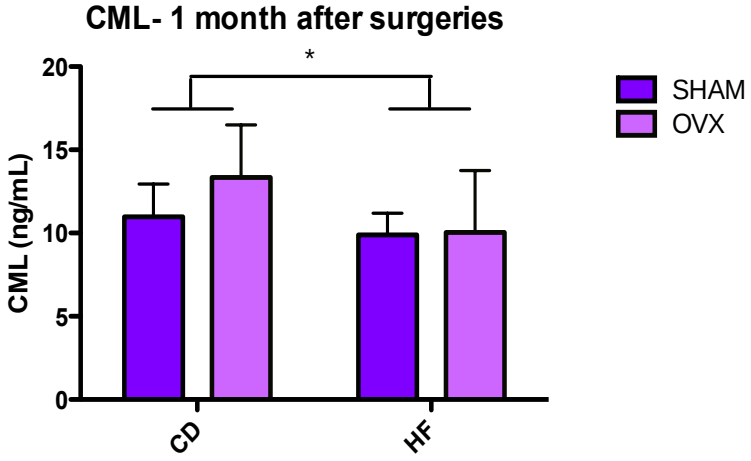
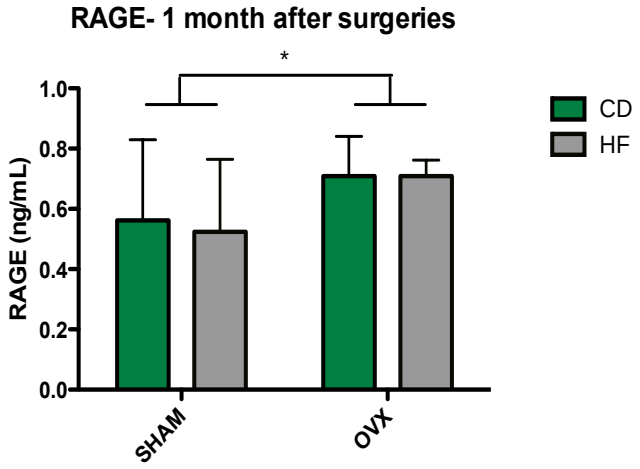


Fig 5. ELISA results for serum RAGE measured one month after surgeries. Mean ± SD, n=9, two-way ANOVA, surgery p=0.015, diet p=0.767, interaction p=0.767.

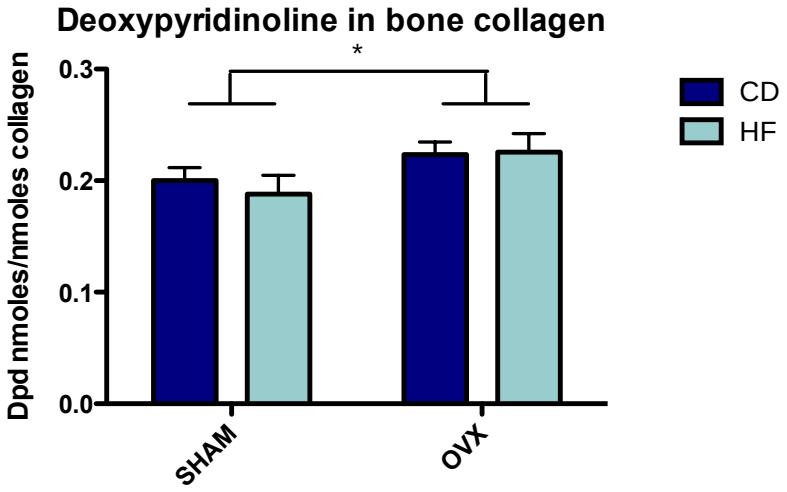


Bone Biochemistry

Method:
Bone pentosidine and normal enzymatic crosslinking contents (pyridinoline and deoxypyridinoline) were measured in acid hydrolysates of the L4 vertebrae using reverse phase high-pressure liquid chromatography.

Results:
Bone pentosidine and pyridinoline crosslinks were present but did not differ between the groups, while deoxypyridinoline was increased in OVX groups, which was a surprising result (Figure 6). Usually following OVX, enzymatic crosslink numbers are decreased while non-enzymatic crosslink numbers are increased, as they use the same binding sites (Saito&Marumo 2010). The presence of increased deoxypyridinoline crosslinks suggests that the neither the diets, nor the surgeries led to an increase in the amount of pentosidine crosslinks in bone collagen AGEs.

Fig 6. HPLC results for bone collagen deoxypyridinoline., mean ± SD, n=9, two-way ANOVA, surgery p=0.002, diet p= 0.590, , interaction p=0.438.

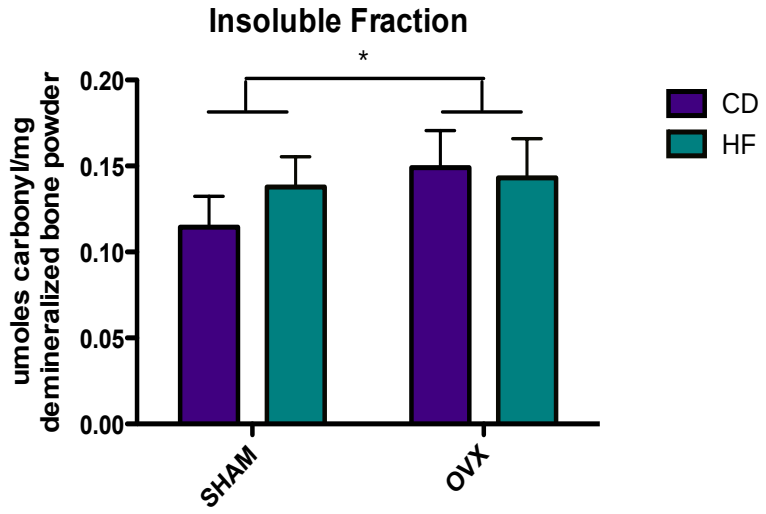


Protein Carbonylation

Method:
Carbonylation of bone collagen (both soluble and insoluble fractions) was investigated as a marker for oxidative damage of bone collagen molecules. L1-L3 vertebrae were demineralized and derived with a 2,4,-dinitrophenylhydrazine solution to measure the presence of carbonyl groups.

Results:
The total carbonyl content and the carbonyl content from the soluble fractions were unchanged in all of the surgical groups. For the insoluble fraction, groups that underwent OVX had significantly more carbonyl content. The OVX surgical groups significantly showed higher carbonyl content in the insoluble fraction, while no differences were noted based on diet (Figure 7). This indicates that the groups that underwent OVX procedures had increased oxidative damage. This is expected in this model, as a cessation of estrogen production leads to increased levels of oxidative stress (Lean *et al.*, 2003). The insoluble, soluble and total carbonyl content also did not differ between either of the groups sacrificed at 6 months of age.

Fig 7. Carbonyl assay results for the insoluble fraction. Mean ± SD, n=9, two-way ANOVA, surgery p=0.007, diet p=0.220, interaction p=0.04



Immunohistochemistry

Method:
The decalcified tibia sections cut when preparing for TRAP analysis were used in this assay. Four five μm serial sections were evaluated, one was stained with TRAP to identify active osteoclasts, one was stained immunohistochemically for RAGE expression and the remaining slides were used as negative controls. The TRAP and immunohistochemistry sections were sequential to ensure that osteoclasts expressed on one slide were the same cells expressed on the subsequent slide. We performed TRAP staining as mentioned previously, and when we observed a TRAP-positive active osteoclast we cross-referenced with the sequential immunohistochemistry stained section to see if the osteoclast also expressed RAGE. This allowed us to co-localize active osteoclasts with RAGE expression. In this assay we measured RAGE expressing osteoclasts (Oc.RAGE), which we normalized to both bone surface and total number of osteoclasts (Oc.RAGE/BS, Oc.RAGE/N.Oc).

Results:
There were no detectable differences in Oc.RAGE/BS nor Oc.RAGE/N.Oc, but we did observe a trend that the groups fed the high fructose diet had increased Oc.RAGE/N.Oc (p=0.08) (Table 7). This suggests that the high fructose diet may have induced more RAGE expression within the active osteoclasts. We can be fairly certain that this trend towards an increase in RAGE is probably not due to accumulation of AGEs from our previous observations. It is possible that this trend towards more RAGE expression was caused by oxidative stress that was inflicted by the high fructose diet.

	HF+OVX	HF+SHAM	CD+OVX	CD+SHAM
Osteoclasts (#)	49.88 ± 26.35	87.5 ± 44.64	56.63 ± 48.55	147.1± 94.12
RAGE expressing osteoclasts (#)	15.5 ± 11.66	23 ± 13.27	10.38 ± 7.6	33.38 ± 21.21
RAGE expressing osteoclasts/bone surface (1/mm)	0.52 ± 0.15	0.46 ± 0.23	0.34 ± 0.13	0.47± 0.21
RAGE expressing osteoclasts/ total number of osteoclasts (%)	0.29 ± 0.12*	0.26 ± 0.05*	0.22 ± 0.1	0.22 ± 0.07

Table 6. TRAP/RAGE co-localization results for surgical groups.
*Values reported as mean ± SD. *Trend (p=0.08) HFD having a greater percentage.*

Summary Table of All The Experiments

Assay	Result
microCT (cortical bone)	No change in microarchitecture, increased vBMD in OVX groups.
microCT (trabecular bone)	vBMD, BV/TV, Tb.N were decreased in OVX groups.
DXA (cortical bone)	No change in BMD or BMC.
DXA (trabecular bone)	BMD was decreased in OVX groups.
H&E stain on liver	No signs of non-alcoholic fatty liver disease.
Decalcified histology (TRAP)	Trend toward HF+OVX interaction having more Oc.S/BS and N.Oc/BS.
Undecalcified histology (Static- Goldner's Trichrome staining)	OVX groups had more OS/BS and OV/BV. HF groups had a greater O.Wi.
Undecalcified histology (Dynamic- Calcein green)	OVX groups had greater dLS, Ir.L.Width, MS/MAR, BFR/BV, BFR/BS.
ELISA (pentosidine)	Serum pentosidine was present in all surgical groups at similar levels.
ELISA (Carboxymethyllysine)	Serum CML was increased one month after surgeries in groups fed the isocaloric control diet.
ELISA (RAGE)	Soluble RAGE was increased one month after surgeries in OVX groups.
HPLC (pentosidine and pyridinoline)	Levels were present at similar levels in all surgical groups.
HPLC (deoxypyridinoline)	Levels were increased in OVX groups.
Protein carbonylation assay	OVX group had most carbonyl content in the insoluble bone collagen fraction.
Immunohistochemistry (RAGE)	Trend toward HF fed rats having more Oc.RAGE/N.Oc.

Conclusion

The goal of the high fructose diet treatment was to characterize the effects of AGEs on bone loss during OVX. It was expected that the high fructose diet would cause an increase in AGEs through initiating the Maillard reaction, which has been seen in previous studies. However, no differences in AGEs were seen between rats fed the high fructose diet and rats fed the control diet. There was no difference in circulating pentosidine or CML, or in the amounts of collagen bound pentosidine, measured directly after high fructose feeding (baseline groups), or 3 months after the diet was discontinued (surgical groups). Therefore, majority of the skeletal changes that were observed were solely due to the OVX treatment, and the results we saw were mostly consistent with previous studies. Macroscopically, we saw that the OVX-treatment lead to more bone loss in trabecular bone while increasing vBMD in cortical bone. At the tissue level, we saw an increase in bone formation parameters suggesting that OVX significantly increased tissue-level bone formation, consistent with previous results of histomorphometry of bone formation in OVX rats. However, no significant changes were seen in bone resorption parameters, which may be because that OVX rats were in the slow phase of bone loss. Finally, we also saw that the OVX-treatment led to more oxidative damage, which is common due to estrogen deficiency leading to oxidative stress.

We also attempted to observe whether soluble and cell-bound RAGE expression was increased following OVX treatment. We found that soluble RAGE seemed to be at an elevated level in OVX groups one month after surgeries, which may have been to combat an upregulation of cell-bound RAGE. No significant changes were found in cell-bound RAGE expression, although the results suggested that groups fed the high fructose diet had more RAGE expressing osteoclasts. This study exhibited trends toward the high fructose diet having an effect on both active osteoclasts, and osteoclast RAGE expression, as both these parameters seemed to be increased, though not significantly, in groups that were fed high fructose diet. These results suggests that the interaction of the high fructose diet with OVX may have caused an increase in osteoclast activity due to an oxidative environment caused by the high fructose diet, leading to more RAGE expression and subsequently increasing the activity of the RAGE expressing osteoclasts. However, since the results were not significant, further study into this subject should be performed before any conclusions are made.

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Opportunities for Training

During the course of this investigation a graduate student was trained in the various techniques under the supervision of the investigators. She received a Master of Science degree at the University of Toronto after successfully defending her thesis. Other people involved were technicians at the division of comparative Medicine at the University of Toronto and post-doctoral fellows and technicians in the Principal Investigator laboratory at the Mount Sinai Research Institute.

4. **Impact**

In the field of post-menopausal osteoporosis, we have introduced a novel hypothesis to understand why some women suffer from fracture post-menopause and others do not. This project was undertaken to examine this hypothesis. While our research provided some insight into the effects of AGE and RAGE on the development of post-menopausal osteoporosis, more research is needed to understand the mechanisms underlying this phenomenon and to prove or disprove our hypothesis. If our hypothesis is ultimately proven to be valid, it will have a great impact on the entire field of osteoporosis research.

Future research into our hypothesis may lead to the development of new biomarkers to predict which women will ultimately suffer from osteoporotic fractures.

Ultimately, this will have a great impact on public health, public policy and in changing the behaviour of people in order to prevent fractures.

5. **Changes / Problems**

Nothing to Report

6. **Products**

Master of Science
Department of Laboratory Medicine and Pathobiology with a collaboration in Musculoskeletal Sciences, University of Toronto
2016
Thesis Title: The role of advanced glycation endproducts in post-menopausal osteoporosis
Supervisor: Drs. Marc Grynpas and Thomas Willett

Peer Review Paper
Willett TL, **Pasquale J**, Grynpas MD. Collagen modifications in post-menopausal osteoporosis: Advanced glycation endproducts may affect bone volume, structure and quality. *Curr Osteoporos Rep*. 2014;12(3):329-337**.**

Poster Presentations
Why do only some women develop post-menopausal osteoporosis?
2014 20th Canadian Connective Tissue Society Conference, London, Ontario
2014 Laboratory Medicine and Pathobiology Graduate Research Conference, University of Toronto
2014 Toronto Musculoskeletal Centre Research Day, University of Toronto
2015 21st Canadian connective tissue conference, Laval Quebec

7. **Participants and Other Collaborating Organizations**

Name:	Drs. Marc Grynpas and Tom Willett
Project Role:	Principal Investigator and Co-Principal Investigator
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	24
Contribution to Project:	Drs. Grynpas and Willett supervised Julie Pasquale and interpreted the results of the experiments and corrected her thesis.
Funding Support:	N/A

Name:	Julia Pasquale
Project Role:	Graduate Student
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	24
Contribution to Project:	Ms. Pasquale conducted the animal experiments at the Department of Comparative Medicine (DCM) under the supervision of Dr. Kate Banks (DVM and Facility Director). She analyzed the bones, interpreted the results and wrote her thesis.
Funding Support:	N/A

Collaborating Organization:
University of Toronto, Department of Comparative Medicine (DCM) where the animal experiment was conducted under the supervision of Dr. Kate Banks (Facility Director).

8. **Special Reporting Requirements**

Nothing to Report

9. **Appendices**

Nothing to Report